A β-1,3-galactosyltransferase and brainiac/bre5 homolog expressed in the midgut did not contribute to a Cry1Ab toxin resistance trait in *Ostrinia nubilalis*

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Abstract

Post-translational glycosylation of midgut epithelial protein and lipid receptors may be required prior to binding of activated *Bacillus thuringiensis* (Bt) Cry toxins. A 931 bp cDNA encoding a putative 297-residue β-1,3-galactosyltransferase (β3GalT5) was cloned from larval *Ostrinia nubilalis* midgut tissue, and showed homology to *Drosophila* brainiac (brn) and *Caenorhabditis elegans* bre5 proteins. Single nucleotide polymorphisms (SNPs) were detected in coding and promoter regions of *O. nubilalis* β3GalT5 (Onb3GalT5), of which 3 of 31 CDS SNPs were non-synonymous. SNPs within *Hae*III and *Msp*I recognition sites were confirmed by PCR–RFLP, and are Mendelian inherited. Analysis of F_2 pedigrees suggested an Onb3GalT5 SNP C660 fixed within a Cry1Ab-resistant colony was not correlated with Cry1Ab resistance traits, as measured by higher larval *O. nubilalis* weights when fed toxin-containing diet.
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1. Introduction

Susceptibility of insects to *Bacillus thuringiensis* (Bt) crystalline (Cry) toxins is orchestrated by events in the alimentary tissue. Activated Cry toxins bind the extracellar domains of aminopeptidase N (APN; Knight et al., 1994), cadherin (Vadlamudi et al., 1993; Francis and Bulla, 1997), and alkaline phosphatase glycoprotein receptors (McNall and Adang 2003; Jurat-Fuentes and Adang, 2004; Jurat-Fuentes et al., 2002). Glycoprotein receptors may be localized in cholesterol-rich lipid rafts (Zhuang et al., 2002), and interaction with Cry toxin is required prior to pore formation that results in larval death (Lorence et al., 1997; Tabashnik, 2001). Carbohydrate modification on membrane-bound protein receptors has been shown to be involved in toxin–receptor interactions (Knowles et al., 1991; Masson et al., 1995), suggesting that activated Cry

toxins may associate with unrelated midgut receptor proteins that share similar carbohydrate modifications (Griffitts et al., 2001). Furthermore, Bt toxin domain III may interact with *N*-acetylgalactosamine (GalNAc) moieties of APN (Jenkins et al., 1999, 2000), with binding enhanced by association with cell membrane phosphatiylcholine (Sangadala et al., 2001) additionally suggesting that toxin binding may require a glycolipid association (Kumaraswami et al., 2001; Griffitts et al., 2005).

Nematicidal toxins Cry5B and Cry14A share structural homology and invoke a similar physiological response as commercially used transgenic Cry1A toxins, suggesting similar modes of action (Griffitts et al., 2001). *Caenorhabditis elegans* is a model organism with extensive genomic information available (The *C. elegans* Sequencing Consortium, 1998). Gut cells from five recessive *C. elegans* Bt resistance (*bre*) mutants failed to take up Cry5B and Cry14A toxins, and evaded membrane pore formation that causes cell lysis (Marroquin et al., 2000; Griffitts et al., 2001). Complementation mapping identified a *C. elegans*

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chromosome region containing a putative β-1,3-galactosyl-transferase family 5 member (β3GalT5; Amado et al., 1999; Griffitts et al., 2001; Hennet, 2002) with homology to *Drosophila melanogaster* BRAINIAC (Panin and Irvine, 1998; Bruckner et al., 2000). Bre5 and brn proteins were shown to include *N*-acetylglucosaminyltransferase activity in *C. elegans* (Griffitts et al., 2003) and *D. melanogaster* (Muller et al., 2002), respectively, which may add terminal GalNAc modifications to lipids and oligosaccharides (Hennet, 2002). The bre mutants demonstrated that reduced function or knockout of post-translational modification pathways could mediate Bt toxin resistance in nematodes.

Isolation and characterization of putative β-1,3-galactosyltransferase genes from Lepidoptera are the first steps for determining roles in Bt resistance traits. Larval feeding by the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), causes economic losses to cultivated corn (Mason et al., 1996). Economic injury caused by the European corn borer has been minimized by foliar applications, formulations of Bt that included the spores and toxins that are the major insecticidal components (McWhorter et al., 1972), and transgenic maize expressing Cry1Ab toxins (Koziel et al., 1993). In 2005, 35% of United States corn acreage was planted with commercial hybrids expressing Cry1Ab toxins (USDA-ERS, 2005). If genetic variance for resistance to Cry1Ab were present in wild populations of *O. nubilalis*, the high adoption rate of Bt corn would not only result in a high selection pressure for resistance, but also increase the potential for *O. nubilalis* populations to respond to the selection.

Glycoprotein receptors of *O. nubilalis* epithelial cells, including a 220-kDa cadherin-like protein, and a 145-kDa or 154-kDa aminopeptidase (APN) isoforms, bind Cry1Ab (Hua et al., 2001). A full-length cadherin cDNA from *O. nubilalis* was shown to have putative *N*-glycosylation sites (Coates et al., 2005), and resultant protein identified as a major midgut receptor (Flanagan et al., 2005). Reduced level of a trypsin transcript T23 was associated with *O. nubilalis* KS-SC colony resistance to native toxins present in Dipel® Bt formulations (Li et al., 2005), but did not show decreased susceptibility to truncated Cry1Ab toxins expressed by transgenic maize (Vaeck et al., 1987). Moderate resistance has been found in *O. nubilalis* laboratory colonies (Bolin et al., 1999; Huang et al., 1999). Molecular tools for monitoring *O. nubilalis* candidate Bt resistance genes, cadherin (Coates et al., 2005) and serine protease genes (Coates et al., 2006), were developed and also shown useful for pedigree and genome mapping applications.

Herein, we report characterization of a unique β-1,3-galactosyltransferase (Onb3GalT5) isolated from larval *O. nubilalis* midgut tissue that also is expressed in head (brain) tissues. Molecular markers for genetic analysis were used to assess the segregation of an *Msp*I PCR–RFLP Onb3GalT5 allele (called SNP 660) that was fixed within a Cry1Ab-resistant colony, with Cry1Ab resistance pheno-types within F_2 progeny originating from resistant female by susceptible male crosses.

2. Materials and methods

2.1. β3GalT5 isolation and characterization

2.1.1. β3GalT5 alignment

Amino acid sequences of *D. melanogaster* CG4934-PA (GenBank NP_476901), *Anopheles gambiae* ENSANG-P00000007774 (GenBank XP_320943), and *C. elegans* BRE-5 (GenBank NM_171420) were aligned using Align X software (Informax, San Francisco, CA; gap penalty = 10). A region of peptide similarity was identified (WYVSLEEYP) and corresponding cDNA sequences used to design a degenerate 3′ RACE oligonucleotide b3GalT5-3pR (5′-TGG TAC GTT TCG YTG GAG GAR TAY CC-3′) with Primer3 (Rozen and Skaletsky, 1998).

2.1.2. Complementary DNA (cDNA) synthesis, cloning, and sequencing

Ten dissected larval *O. nubilalis* midguts were bulked, ground to powder in liquid nitrogen, and RNA extracted with RNeasy extraction kits (Qiagen, Valencia, CA) according to manufacturer instructions. First-strand complementary DNA (cDNA) synthesis used 0.5 μM of a poly(T) adapter (PT-AD; 5′-GGT GTA ATA CGA CGG CCT GGA ATT CTT TTT TTT TTT TTT T-3′), 2 μg of total RNA, and 40 U AMV reverse transcriptase (Promega, Madison, WI) in a 20 μl reaction volume incubated at 42 °C for 30 min on a PTC-100 thermocycler (MJ Research, Watertown, MA). Second-strand cDNA synthesis reaction used a hot start with 1.0 μl first-strand cDNA product, 5 pmol each of primer b3GalT5-3pR and poly(T) adapter core primer (PT-Adc1; 5′-GTG TAA TAC GAC GGC CTG G-3′), 1.0 U *Tli* proofreading polymerase (Promega), 100 μM dNTPs, 1.5 mM MgCl₂, and 2.5 μl of 10× thermal polymerase buffer (Promega) in a 25 μl reaction volume. A PTC-100 thermocycler (MJ Research) performed at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s for 35 cycles. Amplification was confirmed and fragment size estimated by agarose electrophoresis. PCR products were ligated using the pGEM-T vector (Promega), and used to transform *Escherichia coli* SURE cells (Stratagene).

DNA sequencing of plasmid inserts used dye-terminator cycle sequencing (DTCS) quick-start kits (Beckman-Coulter, Fullerton, CA) in 10 μl reactions according to manufacturer instructions with 1.6 pmol T7 primer. Primer extension products were purified by ethanol precipitation, suspended in 40 μl deionized formamide, and separated on a CEQ 8000 Genetic Analysis System (Beckman-Coulter) with method LFR-1.

Oligonucleotide primer Onb3GalT5-R1 (5′-TGC TGC GGC ACT AAG CCC AC-3′) was designed from 3′ RACE cDNA sequence and minimize annealing to the First Choice RNA Ligase-Mediated (RLM) RACE kit 5′

adapter outer primer (5′-GCT GAT GGC GATG AAT GAA CAC TG-3′; Ambion, Austin, TX). First Choice RLM RACE kit (Ambion) was used to isolate 5′ cDNA end of the *O. nubilalis* transcript according to manufacturer instructions. RT-PCR products were ligated, cloned, and sequenced.

2.2. Onb3GalT5 transcription

Larval *O. nubilalis* from the Corn Insects and Crop Genetics Research Unit (CICGRU) laboratory colony were reared on meridic diet from neonate to 5th instar. Total RNA was extracted from whole larvae, midgut, fat body, and head capsule with RNeasy extraction kits (Qiagen) and used a template for RT-PCR. Individual first-strand cDNA reactions used 250 ng total RNA, 10 pmol of primer Onb3GalT5-R1, 2.5 U *Tth* polymerase (Promega), 100 µM dNTPs, 2.5 mM $MnCl_2$, and 1.0 µl of 10× thermal polymerase buffer (Promega) in a 10 µl reaction volume. A PTC-100 thermocycler (MJ Research) performed a primer extension cycle of 85 °C for 1 min, 54 °C for 1 min, and 72 °C for 20 min. A 4.0 µl aliquot of first-strand cDNA synthesis product was mixed with 1.6 µl chelate buffer (Promega), 10 pmol of primer b3GalT5-3pR (5′-TGG TAC GTT TCG YTG GAG GAR TAY CC-3′), $MgCl_2$ to a concentration of 2.0 mM, and water to a final volume of 20 µl. RT-PCR reactions were carried out on a PTC-100 thermocycler (MJ Research) using 35 cycles of 95 °C for 30 s, 57 °C for 20 s, and 72 °C for 20 s. RT-PCR products (20 µl) were separated on a 1 × 20 cm 6% polyacrylaminde (19:1 acrylaminde: bisacrylamide) 1 × tris-borate EDTA gel.

2.3. Onb3GalT5 single nucleotide polymorphism

Genomic DNA was isolated from 13 adult *O. nubilalis* and 2 adult *Ostrinia furnicalis* moths using DNAeasy isolation kit (Qiagen) according to manufacturer directions, and used for comparison of Onb3GalT5 coding sequence (CDS). The Onb3GalT5 CDS was amplified using 2.5 mM $MgCl_2$, 50 µM dNTPs, 7.5 pmol each of primers Onb3GalT5-F1 (5′-CGT GAC AAT GAT GTC GTT CAA-3′) and Onb3GalT5-R1, 0.45 U *Taq* DNA polymerase (Promega), and 100 ng of DNA template in a 12.5 µl reaction. PTC-100 thermocycler conditions used 95 °C for 2.5 min, followed by 40 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. PCR products purified using Qiaquick PCR spin columns (Qiagen), and were sequenced in both directions using 1.6 pmol primer Onb3GalT5-F1 or Onb3GalT5-R1. Sequence data were visually inspected for presence of heterozygotes as overlapping fluorescent label peaks. Alignments were made among genomic and cDNA sequences using AlignX software (Informax; gap penalty = 5), and polymorphic nucleotide sites within restriction endonuclease sites identified. There were no introns in the genomic copy of *O. nubilalis* β-1,3-galactosyltransferase (Onb3GalT5).

Onb3GalT5 genotypic frequencies were determined for individuals from a colony resistant to the Cry1Ab toxin, and wild populations collected from light traps at Mead, NE, and Ames, Hampton, and Hubbard, IA locations. PCR using Onb3GalT5-F and Onb3GalT5-R1 primers were set up as described previously. Individual digest reactions included 7.5 µl of the Onb3GalT5-F1 and Onb3GalT5-R1 primed PCR product, 2.5 µl 10× buffer, 0.1 mg/µl BSA, and 0.25 U of *Msp*I or *Hae*III in 25 µl, and were incubated at 37 °C for 10–16 h. Entire digest volumes separated on 10 cm 2% agarose electrophoresis.

2.4. Onb3GalT5 BAC and 5′-UTR sequence

A pECBAC vector-based bacterial artificial chromosome (BAC) library was constructed from *Eco*RI digested *O. nubilalis* genomic DNA by Amplicon Express (Pullman, WA) and transformed in *E. coli* strain XL1-Blue. A total of 18,432 clones were screened by PCR with primers Onb3GalT5-F and Onb3GalT5-R1 using conditions described in Section 2.3. The Onb3GalT5 locus was PCR amplified from a single BAC clone, OnB-2G17, and confirmed by DNA sequencing. The Onb3GalT5 promoter region and 5′-untranslated region (5′-UTR) were recovered using the TOPO Walker kit (Invitrogen, Carlsbad, CA) according to manufacturer instructions that included primer Onb3GalT5-R1 and 0.1 µg BAC clone OnB-2G17 template. An approximately 1.5 kb fragment was amplified, purified using Qiaquick PCR spin columns (Qiagen), and DNA sequenced as described previously individually using 1.6 pmol of primers Onb3GalT5-R2 (5′-CCT TCT TCG CCA GTA TGC C-3′), Onb3GalT5-5pSeq (5′-ATG CGG CCT GGA ATC AAG TT-3′), and Onb3GalT5-5pSeq (5′-TTG AAC GAC ATC ATT GTC ACG-3′).

2.5. Onb3GalT5 Mendelian inheritance and involvement in Cry1Ab resistance traits

A field-collected colony of *O. nubilalis* has been exposed to laboratory selection for resistance to Cry1Ab since 2003 (>25 generations) at USDA-ARS, CICGRU, Ames, IA. Approximately 70% of neonates from this Cry1Ab-resistant colony (Cry1Ab[R]) survive to ≥2nd instar when fed 20,000 ng Cry1Ab/cm^{2} for 7 days, and are ≥1000-fold greater than its parental control colony (isoline; 50% survival to ≥2nd instar at 6.2 ng Cry1Ab/cm^{2} after 7 days of feeding = L.C. 50). Larval development of Cry1Ab[R] is also less delayed on sublethal doses of Cry1Ab compared to susceptible colonies (Fig. 1), making sublethal doses a viable tool for scoring larval-development phenotypes. Correlation between larval weight after exposure to sublethal doses of Bt toxin (5 and 7.5 ng Cry1Ab/cm^{2}) was made with Onb3GalT5 genotypes and alleles segregating among F_2 progeny. Two *O. nubilalis* F_1 families (Fam 3 and Fam 8) were established via paired matings of a female from the Cry1Ab[R] colony with a Cry1Ab-susceptible male from the CICGRU colony (Cry1Ab[S]). Two paired matings
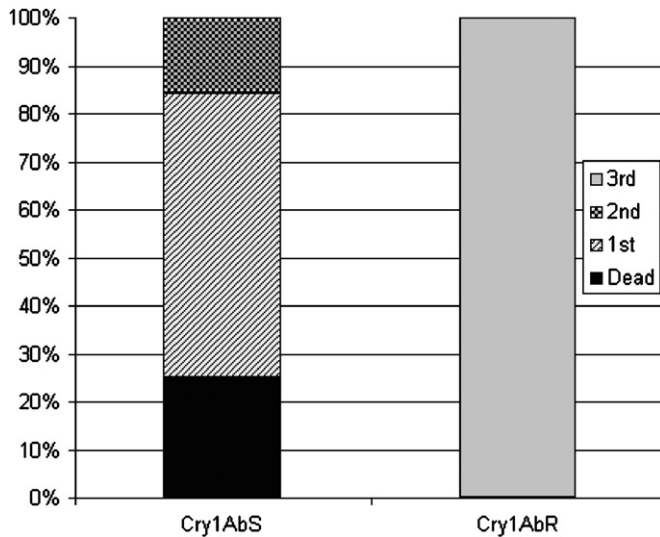
Fig. 1. Developmental differences between larvae from Cry1Ab-susceptible (Cry1Ab^S) and Cry1Ab-resistant (Cry1Ab^R) colonies after 7 days feeding on $5.0\,\text{ng/cm}^2$ Cry1Ab toxin.

of full-sib F_1's were made from each family for a total of four F_2 families (Fam 3-14, Fam 3-15, Fam 8-06, and Fam 8-19). Two doses of Cry1Ab (5 and $7.5\,\text{ng/cm}^2$) were used via the overlay method described by Marçon et al. (1999). F_2 neonates from each family were fed one of the Cry1Ab-overlay diets (100–200 larvae per family), or control (24 larvae).

2.6. Data analysis

The Onb3GalT5 peptides derived from cDNA (AY821558) and BAC clone OnB-2G17 (AY821558) were aligned with homologous *Aedes aegypti* (EAT48595), *A. gambiae* (XP_320943), *Tribolium castaneum* (XP_972668), *D. melanogaster* (NP_476901), *Bombyx mori* (ABF51493), *Homo sapiens* (CAC83093), and *C. elegans* peptides (AAK72094) using AlignX software (Informax). The resulting 365-residue peptide sequence alignment was subjected to Parsimony analysis with 1000 bootstrap iterations performed by the SeqBoot program with *C. elegans* bre5 peptide as the outgroup, followed by ProtPars, then a strict consensus tree estimated from all possible phylogenies with CONSENSE (Felsenstein, 1989) and viewed using TreeView (Page, 1996).

Deviation of observed and expected genotypic frequencies from Hardy–Weinberg Equilibrium (HWE) in the1000-fold Cry1Ab ^{R} colony, control isoline, and Mead, NE, and Ames, Hampton, and Hubbard, IA populations was assessed by chi-square (χ^2) tests (TFPGA software package, Miller, 1997).

F_2 families were used to determine if Onb3GalT5 genotypes exhibited Mendelian inheritance, and also to determine if their segregation was related to larval development on sublethal Cry1Ab diets. Replicated goodness-of-fit tests (Sokal and Rohlf, 1995) were used to determine if Onb3GalT5 genotypes frequencies were

inherited in 1:2:1 ratio (Mendelian expectation). The replicated goodness-of-fit tests produce several G statistics. G_H tests whether the frequencies of Onb3GalT5 genotype are homogeneous across all F_2 families. The pooled-G statistic (G_{Pooled}) tests whether the Onb3GalT5 genotypes pooled across all F_2 families fit a 1:2:1 Mendelain expectation. Finally, the total G (G_{Total}) statistic measures whether the data as a whole fit Mendelian expectations. Analyses were performed separately for genotypic data from control and Cry1Ab diets.

Single-marker regressions were used to test the null hypothesis of no relationship among the segregation of resistance phenotypes (log weight of larvae feeding on Cry1Ab overlay) with Onb3GalT5 genotypes (0, 1, or 2 copies of the M2 allele fixed within the Cry1Ab^R colony) within F_2 families. Regressions were performed using the MIXED procedure of SAS (v. 9.1.3) via restricted-maximum-likelihood methods (Littell et al., 2006). Onb3-GalT5 genotype was the only fixed effect entered into the model. F_2 Family, block nested within an F_2 family, and larvae nested with a block ("Residual" error) were considered random sources of variance in their affects on larval log weight. The relationship between Onb3GalT5 genotypes and log weight was considered significant if $P > 0.05$.

3. Results and discussion

3.1. Onb3GalT5 gene identification and phylogeny

A 931 bp midgut-expressed cDNA was isolated from *O. nubilalis* encoding a single 297-residue protein identified as a putative β-1,3-galactosyl-transferase (GenBank accession no. AY821557; Fig. 2). The gene and transcript was referred to as *O. nubilalis* β-1,3-galactosyl-transferase 5 (Onb3GalT5). Homology searches using BLASTp and PredictProtein (Rost, 1996) identified a conserved galactosyltransferase domain, and high similarity (S) and identity (IDE) to *D. melanogaster* BRAINIAC (GenBank: Q24157, BLASTp score = 149, *E*-value 10^{-35}; S = 54%, IDE = 34%), *C. elegans* BRE-5 (GenBank: NP_741492, BLASTp score = 99, *E*-value 6×10^{-20}), and *B. mori* β-1,3-galactosyltransferase (GenBank: NP_741492, BLASTp score = 320, *E*-value 6×10^{-107}).

PredictProtein identified an *N*-glycosylation site, and semi-conserved DDD and EDVTVG motifs present among β-1,3-galactosyltransferases within the *O. nubilalis* peptide. Seven protein domains, highly conserved among β-1,3-galactosyltransferase family 5 members, also were identified in the proposed Onb3GalT5 peptide (Hennet, 2002). An Onb3GalT5 protein transmembrane region (TMR) was predicted from residues 11 to 27 using the program Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html; total score: 1840). The Onb3GalT5 TMR (LLLCVCVVISVYYLLGI) was similar that of *D. melanogaster* BRAINIAC (LLLRCLLVLPLILLVDYCGL; Fig. 2). Although predictive, cellular localization of Onb3GalT may be in the

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O nubilalis BAC      (1)  MMSFKRRKYKLLLCVCVVISVYYLLGINDYVNARRIEDGFDYPLNMDIQPLLQEVMAGKKPSVPPINYYPYRFLTNSGKC
O nubilalis cDNA     (1)  MMSFKRRKYKLLLCVCVVISVYYLLGINDYVNARRIEDGFDYPLNMDIQPLLQEVMAGKKPSVPPINYYPYRFLTNSGKC
B mori               (1)  M---RRNRYKLIVFALVLVYVYHFFGVDYVQSKNFDSDFNYPLNVDIRPIVQAILDGQKPNVKPINYYPYKFLSNYRQC
D. melanogaster BRN  (1)  MQSKHRKLLLRCLLVLPLILLVDYCGLLTHLHELNFERHFHYPLNDDTG-------SGS-ASSGLDKFAYLRVPSFTAEV
C. elegans BRE5      (1)  MFLCVRILKRKYHELSSFQKLLIFTITIFLLWVLGVVDKFRETSFG-DFSWPLETRNLQLR-----SKFTKYPQCKFSGN

O nubilalis BAC     (81)  NTVEKLDLFIVIKSAMDHFGHRNAVRLTYGKENLIPGRIVKSLFFVGIDESYPKSETQKKIDEEMVQFKDIIQIDFRDNY
O nubilalis cDNA    (81)  NTVEKLDLFIVVKSAMDHFGHRNAVRLTYGQENLIPGRIVKSLFFVGIDESYPKSETQKKIDEEMVQFKDIIQIDFRDNY
B mori              (78)  SVVNKPDLVIIVKSAIDHFGHRDAIRKTYGKP-HVQGYNVKTFFFLGVDNAS--SDVQKNITKEMTEFKDIIQMSFRDSY
D. melanogaster BRN (73)  PVDQPARLTMLIKSAVGNSRRREAIRRTWGYEGRFSDVHLRRVFLLGTAEDS-----EKDVAWESREHGDILQAEFTDAY
C. elegans BRE5     (74)  ---GQKIIIIIIKSSAKNGPMRESVRKTWGVFRMIDGVEVMPIFIVGRVENM---EIMRRIDVESEKYKDILAISDIDSY

                          #
O nubilalis BAC    (161)  YNNTIKTMMSFRWVYEHCNTADYYLFTDDDMYISVNNLLDYVHDKPVPTSTGHGNEQLYAGYVFESTPQRFITSKWRVSL
O nubilalis cDNA   (161)  YNNTIKTMMSFRWVYEHCNTADYYLFTDDDMYISVNNLLDYVHDKPVPTSTGHGNEQLYAGYVFESTPQRFITSKWRVSL
B mori             (155)  FNNTIKTWMSFRWIFQHCAEAQHYLFTDDDMYISVQNLLKYVSDVTTA-SERDG--ILFAGYVFKSAPQRFRSSKWRVSL
D. melanogaster BRN(148)  FNNTLKTMLGMRWASDQFNRSEFYLFVDDDYYVSAKNVLKFLGRG----RQSHQPELLFAGHVFQTSPLRHKFSKWYVSL
C. elegans BRE5    (148)  RNNTLKLFGAIDYAANPNQCSSPDFTFLVDDDYLVHIPNLVKFAKTK-----QKEELVYEGFVFDTSPFRLKIHKHSISL

O nubilalis BAC    (241)  EEYPWSKWPSYVTAGAYVVSNKSMKTMYAGSLFVKHFPFDDVYLGILAKKVGLVPQH----------------------
O nubilalis cDNA   (241)  EEYPWSKWPSYVTAGAYVVSNKSMKTMYAGSLFVKHFPFDDVYLGILAKKVGLVPQH----------------------
B mori             (232)  EEYPWDKWPYVTAGAYVVSNKAMKMLYVGSLFVKHFRFDDIYLGIVAKKMGIVPTHCPHFHFYKKPYER-EVYSDVIAS
D. melanogaster BRN(224)  EEYPFDRWPPYVTAGAFILSQKALRQLYAASVHLPLFRFDDVYLGIVALKAGISLQHCDDFRFHRPAYKGPDSYSSVIAS
C. elegans BRE5    (223)  NEYPFSRYPPYVSAGAVFLTSETIARFRNSIRKLKMFPFDDVFTGILAKTVNVAATHNENFIFWCRRVSQKEWDDGVIAV

O nubilalis BAC    (297)  --------------------
O nubilalis cDNA   (298)  --------------------
B mori             (311)  HGYSNHDELIRVWNEQNAL---
D. melanogaster BRN(304)  HEFGDPEEMTRVWNECRSANYA
C. elegans BRE5    (303)  HGYARKDLEYEYSQLNGFE----
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Fig. 2. Peptide sequence alignment of invertebrate β-1,3-galactosyltransferases homologs in *O. nubilalis* (AAV74410 and AAV74411), *Bombyx mori* (ABF51493), *Drosophila melanogaster* (NP_476901), and *Caenorhabditis elegans* (AAK72094). Transmembrane regions (TMR) are double underlined, and regions conserved among Lepidoptera are highlighted. Conserved D-X-D and variant EDVYVG motifs are underlined, and a conserved *N*-glycosylation site (NNT[L or I]) is enclosed within a box. Gaps within the alignment are indicated as a -.

endoplasmic reticulum (30.4% probability; PSORT II prediction http://psort.nibb.ac.jp/form2.html) or Golgi (26.6% probability). Most eukaryotic galactosyltransferases are anchored within the Golgi (Chappell et al., 1994; Hennet, 2002), but the hamster CGT (ceramide: UDP-galactose galactosyltransferase) protein is found in the endoplasmic reticulum of ovarian (CHO) cells (Sprong et al., 1998). Empirical evidence is required to determine any cellular compartmentalization of the Onb3GalT5 protein. Identification of conserved motifs, and similarity to *D. melanogaster* BRAINIAC and *C. elegans* BRE, suggests that the *O. nubilalis* midgut-expressed cDNA (GenBank AY821557) encodes a family 5 β-1,3-galactosyltransferase-like protein (Hennet, 2002).

A consensus Parsimony tree constructed from a 365 amino acid alignment provided additional evidence for the putative *O. nubilalis* protein classification within the β-1,3-galactosyltransferase family 5. Phylogeny further suggested that two clades are present: (1) Lepidoptera, and (2) Diptera and Coleoptera (branch support 756 of 1000 iterations; Fig. 3). Additionally, *O. nubilalis* sequences were predicted most highly related to the lepidopteran *B. mori* β-1,3-galactosyltransferase family 5 member.

3.2. Onb3GalT5 transcription

Onb3GalT5 transcription was investigated by RT-PCR of a 180 bp product from total RNA sources extracted from 5th instar *O. nubilalis* (midgut, fat body, head, and whole larvae). The Onb3GalT5 RT-PCR product was observed in head-, fat body-, and midgut-derived total RNA (Fig. 4). Relative Onb3GalT5 transcript levels appear highest in head-derived total RNA samples, followed by midgut samples. The decreased band intensity observed from whole larvae RNA likely results from dilution of the target with lower expressing fat body-derived RNA. A more exact comparison of transcript expression levels was not made since real-time quantitative RT-PCR procedures were not performed.

Results suggest expression of the Onb3GalT5 transcript in head (possibly brain) and midgut tissues, and lower levels expressed in the fat body. The pattern of Onb3GalT5 transcriptional activation follows that observed from *brainiac* and *bre*5. Strong expression of *brainiac* and brainiac homologs are observed brain tissue (Vollrath et al., 2001). Therefore, *O. nubilalis* brain tissues may highly the express Onb3GalT5 transcript, but other tissues contained within the head cannot be ruled out. Transcription of Onb3GalT5 in midgut tissues also was observed for *bre*5 proteins in *C. elegans* (Griffitts et al., 2001), where the enzyme was predicted to glycosylate cell membrane lipids and extracellular protein domains (Griffitts et al., 2001, 2003).

3.3. Onb3GalT5 BAC clone and 5'-UTR sequence

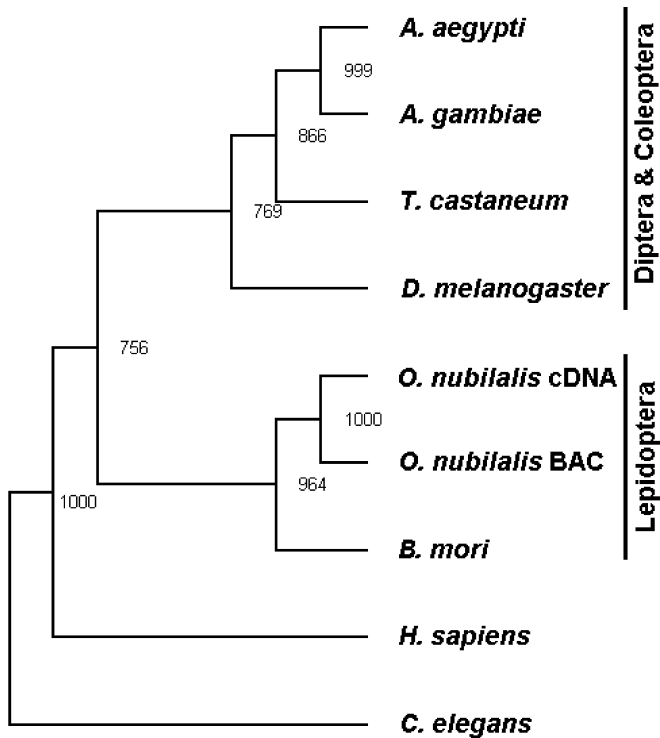Partial sequencing of the BAC clone, OnB-2G17A, generated a 1321 DNA fragment that included the full

Fig. 3. Phylogenetic analysis of *β*-1,3-galactosyltransferase-5 protein sequences. Parsimony method was used to construct a phylogeny from a 365-residue-long peptide alignment. Branch support from 1000 bootstrap iterations is shown at each node. GenBank protein accessions included in the tree; *Aedes aegypti* (EAT48595), *Anopheles gambiae* (XP_320943), *Tribolium castaneum* (XP_972668), *Drosophila melanogaster* (NP_476901), *O. nubilalis* (AAV74410 and AAV74411), *Bombyx mori* (ABF51493), *Homo sapiens* (CAC83093), and *Caenorhabditis elegans* (AAK72094). The *C. elegans* sequence was used as an outgroup to root the tree, and two *β*-1,3-galactosyltransferase-5 clades (Diptera and Coleoptera, and Lepidoptera) are indicated.
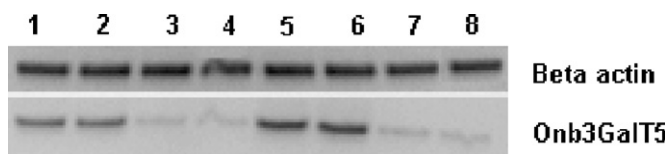

Fig. 4. *O. nubilalis* *β*-1,3-galactosyltransferase (Onb3GalT5) transcript detection by reverse-transcriptase polymerase chain reaction (RT-PCR) from total RNA derived from larval midgut (lanes 1 and 2), fat body (lanes 3 and 4), head (lanes 5 and 6), and whole body (lanes 7 and 8) tissues. The *β*-actin gene was used as a control for RNA integrity.

Onb3GalT5 CDS and a 431 bp portion of the promoter region and 5′-UTR, and was submitted to GenBank (accession AY821558). A potential Onb3GalT5 transcriptional start site (+1; TCAGGTC) was identified 37–44 nt upstream of the ATG start codon, which is similar to the consensus initiator (inr) sequence (YYANWYY). Additionally, an Onb3GalT5 TATA box (AATAAA) may be located at position −18 to −13 (Fig. 5). No other canonical promoter elements were identified. A strong translational start site exists at position +43 (ATGR). Eight nucleotide and one amino acid substitutions were observed in the BAC compared to cDNA sequence (AY821558), suggesting that intraspecific polymorphism exists.

3.4. Onb3GalT5 SNPs and population allele frequency

A strategy of direct DNA sequencing of a 897 bp PCR product was used to identify Onb3GalT5 single nucleotide polymorphisms (SNPs) from *O. nubilalis* and *O. furnicalis* genomic DNA samples. As a consequence of this methodology, PCR products contained a heterogeneous mix of both alleles that simultaneously were detected during DNA sequencing. The strategy was feasible due to absence of introns from genomic DNA, and heterozygous individuals were identified from co-occurring labeled dideoxy-nucleotide signals on electropherograms. Using this method, a total of 38 point mutations (SNPs) were observed from 13 *O. nubilalis* and 2 *O. furnicalis* individuals (30 alleles; data not shown). Intraspecific comparison indicated 31 SNPs were present among a sample of 26 *O. nubilalis* alleles. Three non-synonymous mutations were predicted from DNA sequence alignment, and may result in peptides with three variable residue positions I^{92} or V^{92}, Q^{111} or K^{111}, and D^{215} or N^{215}.

Significant non-synonymous changes on enzymatic functionality remain unknown, but population prevalence can suggest near neutrality. The I^{92} to V^{92} substitution exchanges two residues with hydrophobic short-chain aliphatic side chains, and was predicted from 12 of 26 *O. nubilalis* alleles. Similarly, the D^{215} or N^{215} replacement was observed within 10 of 26 *O. nubilalis* alleles. In contrast, the replacement Q^{111} or K^{111} was predicted only within a single homozygous population sample, and within

Fig. 5. Portion of the *O. nubilalis* *β*-1,3-galactosyltransferase (Onb3GalT5) 5′-untranslated and promoter region sequence from BAC clone OnB-2G17 (GenBank accession no. AY821558). Proposed Onb3GalT5 transcriptional start site is underlined (+1; TCAGGTC; consensus initiator (inr) sequence YYANWYY). A putative Onb3GalT5 TATA box (−16; AATAAA) is double underlined (consensus; TATAAA). Translational start (+43; ATGR) and N-terminal peptide sequence is highlighted. No other canonical promoter elements were identified.

the BAC DNA sequence (AY821558). Although we did not run appropriate experiments, low frequency of the mutation might result in a change in enzymatic function since glutamine (Q^{111}) is uncharged at physiological pH whereas the more rare lysine is positively charged. The point mutation likely is not involved in resistance traits since Q^{111} appears fixed within the 1000-fold Cry1Ab-resistant *O. nubilalis* colony and in 24 of 26 wildtype alleles.

Non-synonymous mutations did not lie within restriction endonuclease recognition site and thus could not be detected by PCR–RFLP. An *Msp*I digest of 897 bp gene fragments (PCR amplified using Onb3GalT5-F1 and Onb3GalT5-R1 primers) detected a C to T SNP located at position 660 of the cDNA sequence (SNP660; AY821557). SNP C660 shows 428, 240, 127, and 92 bp fragments after *Msp*I digestion, whereas a C to T transition in the 3rd codon position of alanine removes the restriction site for SNP T660 (520, 240, and 127 bp RFLP fragments). *Msp*I PCR–RFLP was used to detect the SNP660 among individuals from the Cry1Ab^R colony, its non-selected isoline, and four wild populations. This SNP detection method can show variation at one of 38 potential SNP positions with the Onb3GalT5 gene (position 660 only), and is not comprehensive of all alleles present in the population. In order to differentiate allele in a population, assays for detection of all 38 putative SNPs would be needed. Therefore further discussion will avoid use of allele in the traditional sense, because our *Msp*I digest (4-base cutter) fails to account for nucleotide variation at the remaining 893 base pairs of the Onb3GalT5 PCR fragment. In other words, any given C660 or T660 SNP haplotype could be associated with variation at up to 37 other potential SNP positions and variation at all SNPs would be required to define all allelic types. Chi-square (χ^2) tests indicated SNP ratios based on SNP660 in all wild populations and the non-selected isoline do not deviate significantly from HWE ($P \geqslant 0.1337$). The Cry1Ab^R colony was fixed for SNP C660 (Table 1), suggesting the fixation of the SNP with the selected colony by selection or random genetic drift.

3.5. Onb3GalT5 Mendelian inheritance and larval weights on Cry1Ab diet overlays

The contribution Cry1Ab^R and Cry1Ab^S parents to F_2 pedigrees exploited SNP C660 fixation within the Cry1Ab^R colony. The use of the *Msp*I assay for SNP C660 detection among subsequent F_2 progenies was for identification purposes at a single variable nucleotide position, and not differentiation of all allelic types within each family. Four F_2 pedigrees (Fam 3-14 and 3-15, and Fam 8-09 and Fam 8-19) were derived from two initial Cry1Ab^R ♀ × Cry1Ab^S ♂ parental crosses (Fam 3 and Fam 8). The F_0 parents were screened using *Msp*I PCR–RFLP assays, and all Cry1Ab^R female parents were homozygous for SNP C660, as was observed during HWE estimations (Table 1). Since, all F_0 Cry1Ab^S males were heterozygous SNP C660/T660, thus sharing SNP C660 observed in the Cry1Ab^R colony, all subsequent full-sib F_1 crosses were screened in order to select only matings involving SNP C660/T660 by SNP C660/T660 parents. Heterozygous SNP C660/T660 F_1 parents in Fam 3-14, 3-15, 8-09, and 8-19 allowed determination that the SNP C660 was inherited from the Cry1Ab^R F_0 female parent.

Single-marker regressions were performed to examine the relationship between the segregation of the Onb3-GalT5 SNP at position 660 and the segregation of traits affecting F_2 larval development on control and Cry1Ab bioassays (Fig. 6). Genotypic ratio of F_2 individuals from Fam 3-14, 3-15, 8-09, and 8-19 fed control diet did not deviate from 1:2:1 Mendelian expectations. F_2 families were homogeneous for observed frequencies of Onb3GalT5 SNP genotypes ($G_H = 1.85$, d.f. = 6, $P = 0.933$). Pooled SNP genotype frequencies also fit 1:2:1 Mendelian expectations ($G_{Pool} = 3.59$, d.f. = 2, $P = 0.167$). In addition, the total G statistic indicates that all families were homogeneous for Mendelian expectation of SNP genotypic frequencies ($G_{Total} = 5.43$, d.f. = 8, $P = 0.711$). Similar conclusions were drawn from genotypic data analyzed from F_2 larvae exposed to Cry1Ab overlays ($G_H = 7.32$, d.f. = 6, $P = 0.293$; $G_{Pool} = 3.69$, d.f. = 2,

Table 1
Onb3GalT5 population SNP660 genotyping and Hardy–Weinberg Equilibrium (HWE) test results

Sample	SNP660 genotypes			HWE test results		
	T/T	T/C	C/C	χ^2	*P*-value	d.f.
Cry1Ab^R	0	0	24	0.000	1.000	1
Isoline	6	7	8	2.245	0.1337	1
Mead, NE	5	13	6	0.174	0.6763	1
Ames, IA	11	8	3	0.577	0.4476	1
Hampton, IA	6	11	4	0.069	0.7932	1
Hubbard, IA	5	9	5	0.053	0.8185	1

PCR–RFLP *Msp*I digest defined SNP T660 (520, 240, and 127 bp) and SNP C660 (428, 240, 127, and 92 bp). Samples: 1000-fold Cry1Ab toxin resistant colony (Cry1Ab^R) and its non-selected isoline, Mead, NE, and Ames, Hampton, and Hubbard, IA.
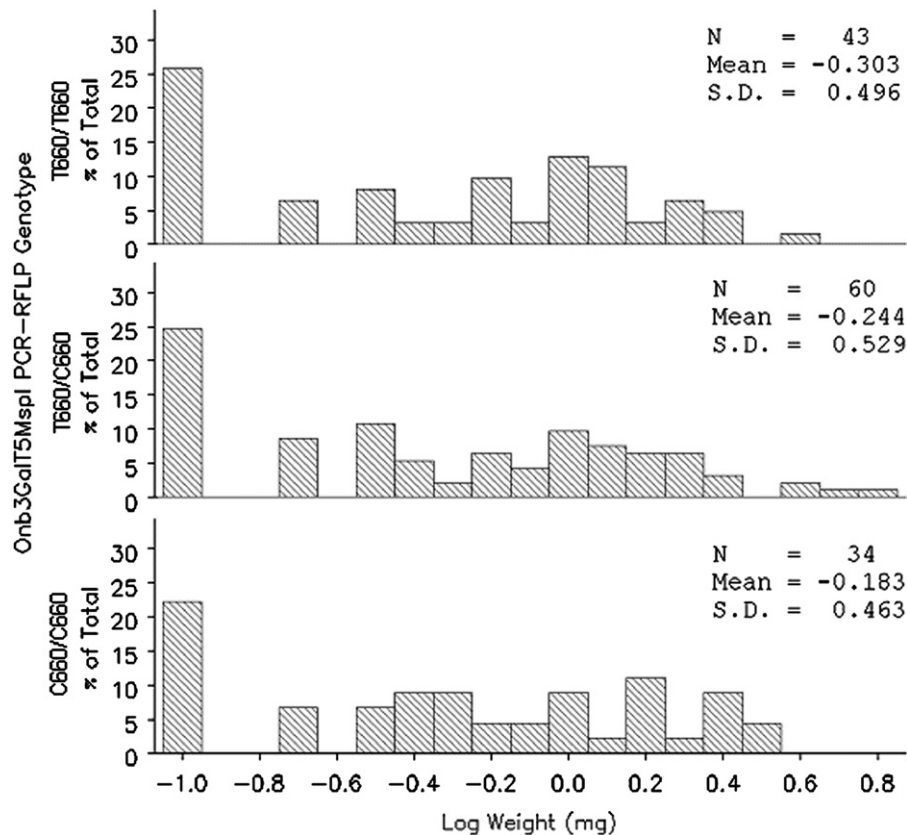
Fig. 6. Histogram of Log_{10} of pooled Fam 3 and Fam 8 F_2 larval *O. nubilalis* weight (mg) fed on 5 mg/ml Cry1Ab toxin diet by Onb3GalT5 *Msp*I PCR–RFLP-defined genotype. SNP T660 was fixed (T660/T660) within the resistant line female parent (see Table 1). Segregation of the M1 allele was not associated with higher Log_{10} F_2 larval *O. nubilalis* weight data for 5 mg Cry1Ab/ml ($F = 2.70$, d.f. $= 1$, $P = 0.106$). An analogous pooled analysis of Fam 3 and Fam 8 F_2 larval *O. nubilalis* weights when fed 7.5 mg Cry1Ab/cm^2 yielded similar results ($F = 0.81$, d.f. $= 1$, $P = 0.369$; histogram not shown).

$P = 0.158$; $G_{Total} = 11.01$, d.f. $= 8$, $P = 0.201$). Mendelian inheritance of the Onb3GalT5 SNP C660/T660 suggests appropriate use in population genetic and mapping experiments.

Single-marker regressions tested the null hypothesis of no relationship between the segregation of resistance phenotypes (log weight of larvae fed on Cry1Ab overlays) with the Onb3GalT5 SNP C660/T660 marker (0, 1, or 2 copies of the C660 SNP fixed within the Cry1Ab^R colony) within F_2 families. The two doses of Cry1Ab (5.0 and 7.5 ng/ml) exhibited negligible differences for larval development. Because two F_2 families were exposed to a single dose of Cry1Ab, we included F_2 family as a random effect in the model and included larval log weights from both diets in analysis. Examination of variance estimates for random effects support the pooling of data across doses (percentages of the total variance for family, replicates within a family, and within family were, respectively, 6.1%, 9.6%, and 84.3%). Larval log weights of F_2 progeny were not dependent on the number of alleles (defined at a single SNP position) originating from their Cry1Ab^R grand-parent ($F = 0.005$, d.f. $= 1$, 133, $P = 0.947$). The growth of F_2 larvae was not related to the segregation of Onb3GalT5 genotypes.

3.6. Conclusions

This research comprises the first report of a lepidopteran β-1,3-galactosyltransferase gene and analysis of its contributions toward Cry1Ab resistance traits. Onb3GalT5 shows homology to β-1,3-galactosyltransferase family 5 genes, and is expressed in larval gut and brain tissues. A synonymous SNP mutation at position 660 of the Onb3GalT5 cds is detected by an *Msp*I PCR–RFLP assay, and SNP C660 is fixed within a Cry1Ab^R colony. Two Cry1Ab^R ♀ × Cry1Ab^S ♂ F_2 pedigrees independently suggested lack relationship between the segregation of Onb3GalT5 alleles affect on F_2 development (larval weight) in Cry1Ab bioassays. Results suggested that the Onb3GalT5 locus or linked genomic regions might not have a significant influence on Cry1Ab resistance traits in the pedigrees analyzed.

If a trait with a strong genetic component and several genes influencing the resultant phenotype, a quantitative trait locus (QTL) type of analysis could be used to partition proportions of the phenotype contributed by individual loci (genes). Percentage contribution of Onb3GalT5 to the *O. nubilalis* Cry1Ab resistance phenotype was not measurable by our experiments or statistical analysis, but could be

elucidated by QTL analysis incorporating Onb3GalT5 and other genomic markers. Independent mechanisms may evolve for resistance to different Cry toxins (Jurat-Fuentes et al., 2003), suggesting alternatively that the Onb3GalT5 marker may be used in diagnosis of Cry1F or Cry2 toxin resistance traits. Additional experiments using other candidate resistance genes, colonies resistant to other Cry toxins, or genome scans followed by detection of contributing QTL will be required to elucidate genetic components of *O. nubilalis* toxin resistance phenotypes.

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